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INDUCTION OF CALCIUM FLUX AND ENHANCEMENT OF CYTOLYTIC ACTIVITY IN NATURAL KILLER CELLS BY CROSS-LINKING OF THE SHEEP ERYTHROCYTE BINDING PROTEIN (CD2) AND THE Fc-RECEPTOR (CD16)[1]

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Binding of the anti-cluster of differentiation (CD) 2 monoclonal antibody 9-1 causes an increase in the concentration of cytoplasmic-free calcium ($[Ca^{2+}]i$) in cultured $CD3^-/CD16^+$ natural killer (NK) cells. This response did not occur in cultured $CD3^+/CD16^-$ cytotoxic T lymphocytes (CTL). Anti-CD16 antibodies could partially block the calcium response when NK cells were stimulated with intact antibody 9-1, and antigen-binding fragment $F(ab')_2$ of antibody 9-1 did not produce a calcium response. Thus an interaction of the 9-1 antibody with CD16 Fc receptors was required for the functional effect. The dual interaction of antibody 9-1 with both CD2 and CD16 was demonstrated by comodulation experiments. The cytolytic activity of cultured NK cells was increased by antibody 9-1 but not by $F(ab')_2$ fragments of antibody 9-1. The enhanced lytic activity was blocked by anti-CD16 antibody, anti-CD18 antibody, and anti-CD2 antibodies that do not block the binding of antibody 9-1. This pattern was distinct from antibody-dependent cell-mediated cytotoxicity which was blocked only by the anti-CD16 antibody. Thus antibody 9-1 enhanced cytotoxicity by activating effector cells. There was no enhancement of lytic activity when $F(ab')_2$ of antibody 9-1 were cross-linked with a polyclonal antiglobulin, even though $[Ca^{2+}]i$ was increased. These results show that induction of a $[Ca^{2+}]i$ response is not sufficient to enhance lytic activity in NK cells, and suggest that signals delivered through CD16 are necessary.

Target cell lysis by cytotoxic T lymphocytes (CTL)[3] and natural killer (NK) cells is initiated by the formation of stable conjugates between effector and target cells, an antigen-independent process which utilizes the sheep erythrocyte receptor (cluster designation (CD) 2) and the LFA-1 complex (CD11, CD18) as adhesion structures (1–4). Thereafter, effectors are programmed for lysis through a Ca^{2+}-dependent mechanism: intracellular microtubules are reorganized and cytoplasmic granules are oriented toward the target cells (5). Granule contents are secreted with release of a complex of proteins, including perforins and serine-esterases, which produce target cell lysis (5, 6). In the case of CTL, induction of lytic activity requires interaction between the T cell receptor/CD3 complex and specific antigen, with triggering of a transmembrane calcium flux and activation of protein kinase C (1, 7–10). On NK cells, a surface receptor capable of inducing cytolytic activity has not yet been identified (11).

Receptor ligand interactions involving CD2 not only facilitate conjugate formation but also appear to be involved in activation of effector cells (12, 13). Binding of certain anti-CD2 antibodies can induce an increase in the concentration of intracellular-free calcium ($[Ca^{2+}]i$) (14). In a previous study, we showed that simultaneous binding of anti-CD2 antibodies 9.6 and 9-1 to resting T cells causes an increase in $[Ca^{2+}]i$ (15). An unexpected finding was the observation that the binding of antibody 9-1 alone was sufficient to trigger a calcium flux in resting $CD16^+$ cells. These findings suggested that activation triggered by anti-CD2 antibodies might have different mechanisms in $CD3^+$ T cells population and in $CD16^+$ large granular lymphocytes (LGL), a population known to contain NK cells. In the present study, we demonstrate that the increased $[Ca^{2+}]i$ induced in $CD16^+$ cells by antibody 9-1, an IgG3 immunoglobulin, requires an interaction with Fc receptors and that cross-linking of CD2 molecules and Fc receptors by the 9-1 antibody activates the lytic program of NK cells.

MATERIALS AND METHODS

Cell preparations. Mononuclear cells were separated from heparinized peripheral blood of healthy human subjects by density-gradient centrifugation on Ficoll-Hypaque (S.G. 1.077). For some exper-

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[3] Abbreviations used in this paper: CTL, cytotoxic T lymphocytes; CD, cluster of differentiation; PBL, peripheral blood lymphocytes; LGL, large granular lymphocytes; ADCC, antibody-dependent cell-mediated cytotoxicity; NK, natural killer; $[Ca^{2+}]i$, concentration of cytoplasmic ionized free calcium; IL-2, interleukin 2.

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iments, monocytes and B cells were depleted by sequential adherence to plastic plates and nylon wool as previously described (16).

Cultured cytotoxic effector cells. Peripheral blood lymphocytes (PBL) were cloned by limiting dilution at a concentration of 0.5-1 cell/well in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 25 U/ml recombinant interleukin 2 (IL-2) (Amgen, Thousand Oaks, CA), 10% pooled human serum (Pel-Freez, Brown Deer, WI), 3×10^4 irradiated (3600 rad) autologous PBL/well and 5×10^3 irradiated (7000 rad) autologous Epstein-Barr virus (EBV) transformed B cells/well. After 21 days, cultures showing >20% specific lysis of K562 cells at a 10:1 effector to target ratio were selected for continued propagation. Cell lines EB4.19 and EB4.3 have an identical $CD2^+/CD3^-/CD8^+/CD4^-/CD11^+/CD16^+/Leu7^+/Leu19^+$ cell-surface phenotype (17, 18). Both showed a germline configuration of the T cell receptor β and γ genes (data not shown). Expression of functional Fc receptors was documented by antibody-dependent cell-mediated cytotoxicity (ADCC) assays by using a Thy-1.1$^+$ SL2 murine thymoma cell line and anti-Thy-1.1 murine monoclonal antibodies (19). Spontaneous cytolytic activity was present against K562 and MOLT-4 cell lines, but not against DAUDI and B lymphoblastoid cell lines "JH" and "DY." On the basis of their function and phenotype, cells of lines EB4.19 and EB4.3 will be termed NK cells. Cell line C3 has a $CD2^+/CD3^+/CD8^+/CD4^-/CD11^+/CD16^-$ phenotype and shows promiscuous cytotoxicity against K562, MOLT-4, DAUDI, and NALM-1 cell lines. This cytolytic activity is not specific for or restricted by major histocompatibility complex (MHC) gene products. Cell line C3 is not active in ADCC assays. In view of the expression of CD3 and the absence of CD16, cells of this line will be termed CTL.

Other CTL cell lines were generated by two sequential primings of normal PBL with allogeneic stimulators followed by culture at limiting dilution. CTL line 4.47 has a $CD2^+/CD3^+/CD8^+/CD16^-$ phenotype and is specific for HLA-B27. CTL line 52 has a $CD2^+/CD3^+/CD4^+/CD16^-$ phenotype and is specific for an antigen expressed by EBV-transformed cells and restricted by HLA-Dw1. Cell lines were maintained by weekly feeding.

Cell lines. The erythroleukemia cell line K562, the pre-B cell line NALM-1, the B cell line DAUDI, and the T cell line MOLT-4 were obtained from American Type Culture Collection (Rockville, MD). The B-lymphoblastoid cell lines "JH" and "DY" were produced by EBV transformation of B cells from healthy volunteers. The lung carcinoma cell line 2981 has been described previously (20). All cell lines were maintained under standard culture conditions in RPMI 1640 medium (GIBCO), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 1 mM pyruvate, and 2 mM glutamine.

Monoclonal antibodies. Murine monoclonal antibody 9-1 was a gift of Dr. Bo Dupont (Memorial Sloan Kettering Cancer Center, New York, NY). Antibody 9-1, which was clustered with CD2 in the Second International Workshop on Leukocyte Differentiation Antigens (21), does not inhibit rosette formation with sheep erythrocytes (22). Immunoprecipitation experiments showed that antibody 9-1 reacts with a 50-kDa polypeptide species that could be precleared by CD2 antibody 35.1 (data not shown). Immunoblotting of human thymocyte lysate showed that antibodies 9-1, 35.1, and 9.6 all bind to molecular species that migrate as a broad band in the 50-kDa region of the gel (data not shown). Ouchterlony double immune diffusion with purified rabbit anti-mouse immunoglobulin (Ig) heavy chain antisera (Zymed) showed that antibody 9-1 has an IgG3 isotype. Antibody-binding fragments $F(ab')_2$ of antibody 9-1 were prepared by papain cleavage at pH 4.5 as described (23). Undigested antibody was removed by chromatography on a protein A-Sepharose column (Pharmacia, Piscataway, NJ) and was not detected in gel electrophoresis analysis of the final $F(ab')_2$ preparation. Binding activity of 9-1 $F(ab')_2$ was documented by indirect immunofluorescence assays, showing that saturation was achieved at concentrations as low as 3 μg/ml. $F(ab')_2$ of antibody 9-1 were conjugated to biotin by mixing 1 ml of 0.1 M $NaHCO_3$ containing 1 mg of protein with 50 μl of dimethyl sulfoxide containing 50 μg Biotinyl-*N*-hydroxysuccinimide (Sigma), and incubation for 3 hr at room temperature. The solution was dialyzed in phosphate-buffered saline and filter-sterilized before use. Characterization of CD2 antibodies 9.6 (IgG2a) and 35.1 (IgG2a) has been described previously (24). Antibodies FC-1 (IgM), and FC-2 (IgG2b) (donated by Dr. Edward Clark, University of Washington) are specific for the CD16 Fc-receptor of LGL and can block Fc binding and ADCC (25). Antibody 31C5.31 (IgG3) (provided by Dr. Irwin D. Bernstein, Fred Hutchinson Cancer Research Center) recognizes the Thy-1.1 antigen of murine thymocytes and does not cross-react with human cells. Antibody 60.5 (IgG3) reacts with a monomorphic determinant of HLA-class I molecules. Antibody 60.3 (IgG2a) is specific for CDw18, the β-chain of the human LFA-1 complex (26). Antibody G1-14 is specific for CD45, the T200 complex. Antibody L6 (IgG2a) recognizes a carbohydrate antigen expressed on lung cell and other carcinomas (18). Rat monoclonal antibody 187.1 is specific for the mouse immunoglobulin κ-chain (27).

Monoclonal antibodies were purified from ascites fluids by chromatography on protein A-Sepharose or by precipitation with 50% saturated ammonium sulfate followed by DEAE-Sephacyl chromatography (Pharmacia). Purified antibodies were dialyzed against phosphate-buffered saline and filter-sterilized. Before each experiment, antibodies were centrifuged (122,000 × G) for 20 min in a Beckman airfuge to remove aggregates. Certain antibodies were conjugated to fluorescein isothiocyanate according to the method of Goding (28).

Immunofluorescence analysis. Cells were incubated with antibody at saturating concentrations and stained by indirect immunofluorescence with the use of affinity-purified fluorescein-conjugated goat anti-mouse Ig antibodies (Tago, Burlingame, CA). A specificity control was provided by staining cells with an isotype-matched antibody of an irrelevant specificity. For competitive binding inhibition experiments, 5×10^4 cells were incubated at 4°C in 25 μl of medium containing 0.1% sodium azide and a saturating concentration of the test antibody. After 30 min, 25 μl of fluorescein isothiocyanate-conjugated antibody was added, and the incubation was continued for another 30 min. Cells were then washed and fixed in 1% paraformaldehyde. For modulation experiments, a similar procedure was followed except that the first incubation was performed for 1 hr at 37°C in absence of sodium azide. Cells were analyzed by flow microfluorimetry with logarithmic amplification of the fluorescence signal.

Measurement of cytoplasmic-free calcium $[Ca^{2+}]i$. In some experiments, the concentration of cytoplasmic-free calcium $[Ca^{2+}]i$ was measured in Quin 2/AM (Calbiochem, San Diego, CA) -loaded cells as previously described (15) with the assay calibrated by the method of Tsien et al. (29). In other experiments, $[Ca^{2+}]i$ was measured in Indo-1 loaded cells by flow cytometry as described in detail elsewhere (30). The Indo-1 ratio of violet to blue fluorescence is directly related to the $[Ca^{2+}]i$ and was digitally calculated in real time for each individual cell by using a liner scale. At the beginning of each experiment, the blue and violet photomultiplier settings were adjusted so that the ratio was 1 for resting PBL. The $[Ca^{2+}]i$ can be calculated from the Indo-1 violet/blue ratio by using the formula derived by Grynkiewicz et al. (31): $[Ca^{2+}]i = K_d \cdot (R - R_{min})/(R_{max} - R) \cdot (f2)/(b2)$, where $[Ca^{2+}]i$ = intracellular ionized calcium concentration (nanomolar); K_d = 250 nm for the intracellular dye; R = Indo-1 violet/blue ratio; R_{min} = Indo-1 violet/blue ratio of calcium-free dye; R_{max} = Indo-1 violet-blue ratio of calcium saturated dye; $f2$ = blue fluorescence intensity of calcium-free dye; and $b2$ = blue fluorescence intensity of calcium-saturated dye. We have previously determined the constants for this system (30). Ratios of 1, 2, 3, and 4 correspond to a $[Ca^{2+}]i$ of 131, 338, 679, and 1346 nM, respectively. The $[Ca^{2+}]i$ of resting T cells was 131 ± 8 (mean ± SD). Data were collected on the 2150 "time mode," and the mean Indo-1 violet/blue fluorescence ratio was calculated and plotted as a function of time.

Cell-mediated cytotoxicity assay. The medium used for the cytotoxicity assay was RPMI 1640 containing 10% fetal calf serum (Hyclone) heat-inactivated for 1 hr at 56°C. Target cells (1.0×10^6) were incubated at 37°C for 1 hr in 150 μl RPMI 1640 containing 300 μCi sodium (^{51}Cr) chromate and 15% fetal calf serum and then washed. In some experiments, ^{51}Cr-labeled cells were preincubated with medium containing monoclonal antibody for 30 min at 4°C, washed, and resuspended in standard medium before the cytotoxicity assay. In other experiments, the cells were dispensed in microwells and antibody was added subsequently. The cytolytic assay was performed by adding effector cells to 1×10^3 ^{51}Cr-labeled target cells in V-bottomed microwells (Linbro) containing a final assay volume of 200 μl. After 4 hr of incubation at 37°C in 5% CO_2, 100 μl of cell-free supernate were collected from each microwell for measurement of ^{51}Cr release. Controls with no antibody and no effectors were used to measure spontaneous ^{51}Cr release. The lysate of target cells treated with 5% NP-40 was used to calculate maximum release. Percent specific release was calculated according to the formula: % specific release = (test − spontaneous)/(maximum − spontaneous) × 100%.

RESULTS

Changes in $[Ca^{2+}]i$ induced by CD2 antibodies. Activation induced by CD2 antibodies was examined by measuring changes in $[Ca^{2+}]i$ in cultured $CD3^-/CD16^+$ NK cell lines and $CD3^+/CD16^-$ CTL cell lines. The $[Ca^{2+}]i$ of NK cells increased immediately after binding of antibody 9-

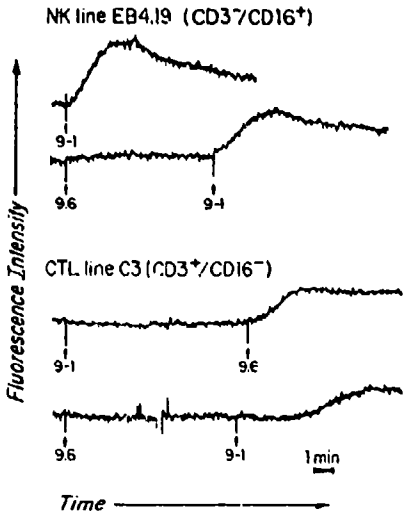

Figure 1. Induction of calcium flux by anti-CD2 monoclonal antibodies 9-1 and 9.6. Cells were loaded with Quin2/AM and assayed for intracellular calcium flux by determining changes in fluorescence intensity. Antibodies were added at the times indicated by *arrows*, at a final concentration of 10 μg/ml.

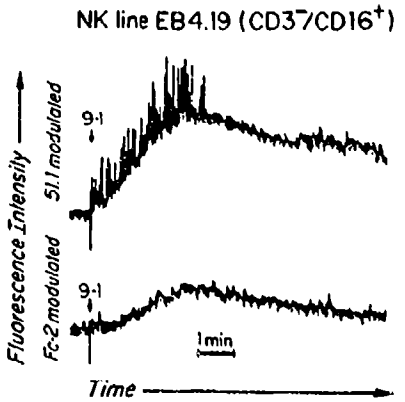

Figure 2. Blocking effect of anti-CD16 antibody FC-2 on calcium flux induced by anti-CD2 monoclonal antibody 9-1. Cells were loaded with Quin2/AM and assayed for intracellular calcium flux by determining changes in fluorescence intensity. Cells were preincubated for 1 hr with saturating concentrations of antibody FC-2 (anti-CD16) or 51.1 (anti-CD8) and then stimulated by antibody 9-1 (10 μg/ml). Antibodies FC-2 and 51.1 had no effect on $[Ca^{2+}]i$ (not shown). The spikes on the CD8-treated cells are artifacts from bubbles.

1 but was not affected by antibody 9.6 (Fig. 1). The increased $[Ca^{2+}]i$ induced by antibody 9-1 reached a maximum level within 2 min and then decreased within 4 min. The $[Ca^{2+}]i$ of the CTL line was not affected by binding of either antibody 9-1 or 9.6 alone (Fig. 1). When CTL were preincubated with antibody 9-1, the addition of antibody 9.6 induced an increase in $[Ca^{2+}]i$ after a latent period lasting less than 1 min with a peak level reached after approximately 2 min. When CTL were preincubated with antibody 9.6, antibody 9-1 was also able to induce an increase in $[Ca^{2+}]i$. In this case the latent period was approximately 3 min and the peak response was reached after approximately 6 min.

Valency requirements for activation of NK cells by CD2 antibodies. Previous studies have shown that CD16, the low affinity Fc receptor known to be expressed on NK cells and granulocytes, has a preferential avidity for murine antibodies of the IgG3 subclass (19). Furthermore, when used alone, the IgG3 antibody 9-1 was able to evoke a calcium flux only in $CD16^+$ cells (15). Thus it was of interest to determine whether its ability to activate NK cells required an interaction with Fc receptors. When cultured NK cells were preincubated with the anti-CD16 antibody FC-2, there was a reduced $[Ca^{2+}]i$ response after stimulation with antibody 9-1 (Fig. 2). Furthermore, NK cells showed no $[Ca^{2+}]i$ increase after stimulation with $F(ab')_2$ of antibody 9-1 (Fig. 3, dashed line [- - - -], at time 0). On the other hand, cross-linking of surface-bound 9-1 $F(ab')_2$ with a goat anti-mouse Ig antiserum caused a prompt increase in $[Ca^{2+}]i$ (Fig. 3, dashed line [- - - -], at 2.5 min). A smaller but definite increase in $[Ca^{2+}]i$ was generated by avidin cross-linking biotin-conjugated $F(ab')_2$ of antibody 9-1 (Fig. 4). Cross-linking of surface-bound antibody 9.6 also induced a calcium flux (Fig. 3). Binding and cross-linking of the control anti-T200 antibody G1-14 had no effect on $[Ca^{2+}]i$ (Figs. 3 and 4). Finally, binding of the IgG3 isotype control anti-HLA class I antibody 60.5 had no effect on $[Ca^{2+}]i$ (data not shown). These data indicate that either multivalent cross-linking of CD2 molecules or formation of complexes

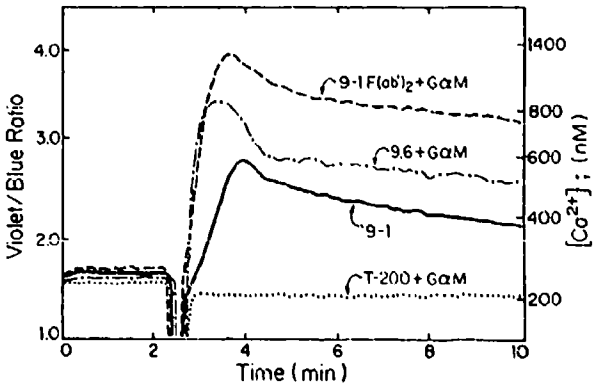

Figure 3. Concentration of $[Ca^{2+}]i$ in cultured NK cell line loaded with Indo-1. The mean Indo-1 violet/blue ratio is shown on the ordinate and a value of 1.0 corresponds to a $[Ca^{2+}]i$ of 131 nM observed in resting lymphocytes. Before any antibody was added to EB4.19 cells, $[Ca^{2+}]i$ was 263 nM. Cells were incubated with antibody 9-1 $F(ab')_2$ (- - - -), antibody 9.6 (— - —), or antibody G1-14(T200) (· · · ·) at time 0, and goat anti-mouse Ig antiserum (GαM) was added after 2.5 min. Antibody 9-1 (——) was added after 2.5 min. Monoclonal antibodies were used at a concentration of 50 μg/ml and GαM at 1/10 dilution.

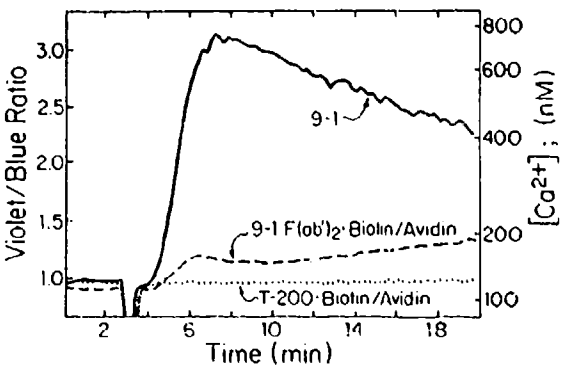

Figure 4. Concentration of $[Ca^{2+}]i$ in cultured NK cell line loaded with Indo-1. The mean Indo-1 violet/blue ratio is shown on the ordinate and a value of 1.0 corresponds to a $[Ca^{2+}]i$ of 131 nM observed in resting lymphocytes. Cells were preincubated for 15 min with biotin-conjugated $F(ab')_2$ of antibody (- - - -) or antibody G 1-14 (T200) (· · · ·) and analyzed at time 0. Avidin was added to cells at 10 μg/ml after 2.5 min. Antibody 9-1 (——) was added after 2.5 min. Antibodies or fragments were used at a concentration of 50 μg/ml.

between CD2 and CD16 are sufficient to trigger an increased $[Ca^{2+}]i$ in cultured NK cells.

We have considered the possibility that antibody 9-1 produced intercellular bridging by binding to Fc receptor on one cell and to CD2 on another. However, the light scatter characteristics of the cells analyzed by flow cytometry did not change after binding of antibody 9-1. This finding excludes that cellular aggregates are formed and supports the alternative hypothesis of a surface cross-link on a single cell.

Modulation of CD16 induced by a CD2 antibody. The dual interaction of antibody 9-1 with both CD2 and CD16 was further demonstrated by comodulation experiments. Preincubation of cultured NK cells at 4°C with antibody 9-1 did not inhibit binding of the anti-CD16 antibody FC-2 and, similarly, preincubation with antibody FC-2 did not inhibit binding of antibody 9-1 (Fig. 5). However, incubation with antibody 9-1 for 1 hr at 37°C caused nearly complete modulation of CD16 whereas incubation with antibody FC-2 had no effect on CD2 expression. Modulation of CD16 was not observed after incubation with the IgG2a anti-CD2 antibody 9.6, with $F(ab')_2$ of antibody 9-1, or with a control IgG3 antibody 60.5, specific for HLA class I molecules.

Activation of the lytic program in NK cells by a CD2 antibody. Given that CD2 has a functional role in lymphocyte activation and that Fc receptors are involved in antibody-dependent target-specific lysis by killer lymphocytes, it was of interest to determine the effect of antibody 9-1 on cytolytic effector cell function. In the presence of antibody 9-1, the lytic activity of the NK cell lines EB4.19 and EB4.3 tested against NALM-1 or K562 targets was profoundly enhanced (Fig. 6). In contrast, and consistent with previous studies, anti-CD2 antibody 9.6 (IgG2a) had an inhibitory effect on NK lytic activity (24, 32). Binding of IgG3 isotype control anti-HLA class I antibody 60.5 had no effect. Antibody 9-1, however, had no effect on the lytic activity the $CD3^+/CD16^-$ CTL line C3 (Fig. 6). Similarly, there was no induction of promiscuous killing or inhibition of lectin-facilitated cytotoxicity

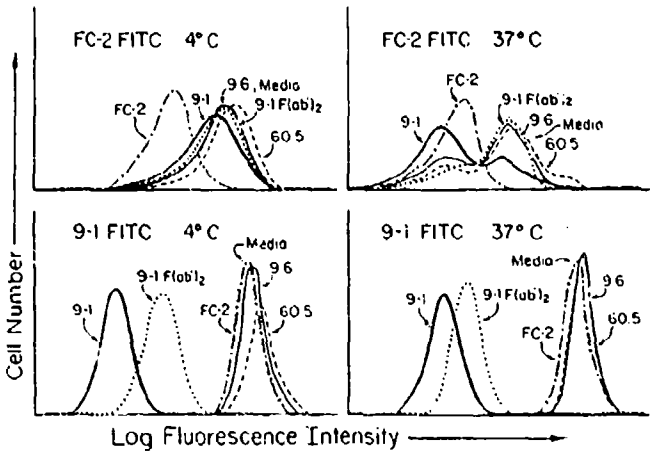

Figure 5. Comodulation of CD2 and the CD16 Fc receptor by antibody 9-1. Cells of the NK cell line EB4.19 were preincubated with a saturating concentration of each indicated antibody or medium for 1 hr at either 4°C or 37°C and aliquots were then stained with fluorescein-conjugated anti-CD16 antibody FC-2 or with fluorescein-conjugated anti-CD2 antibody 9-1. Cells were analyzed by flow microfluorimetry with logarithmic amplification of the fluorescence signal. Antibody 60.5 (IgG3) is specific for HLA-class I molecules.

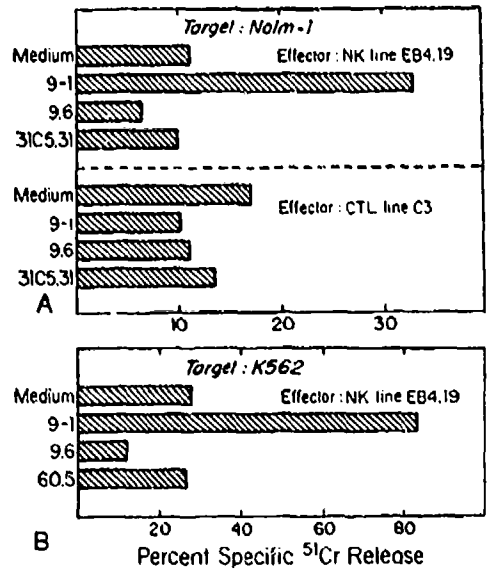

Figure 6. Enhancement of cytolytic activity of a $CD16^+$ cloned NK cell line by antibody 9-1. *A*, Cell line EB4.19 (phenotype: $CD2^+/CD3^-/CD16^+$) and cell line C3 (phenotype: $CD2^+/CD3^+/CD16^-$) were incubated with ^{51}Cr-labeled NALM-1 cells at a 10:1 effector to target ratio for 4 hr at 37°C either in medium or in antibody at a concentration of 1 μg/ml. Incubation of target cells with antibody alone did not increase spontaneous release. Antibody 31C5.31 (IgG3) is specific for murine Thy-1.1 does not react with either EB4.19 or NALM-1 cells. Lytic activity of another $CD3^-/CD16^+$ cell line (EB4.3) was also enhanced by antibody 9-1 (not shown). *B*, Cells from the NK line EB4.19 were incubated with ^{51}Cr-labeled K562 cells (effector to target ratio of 10/1) 4 hr at 37°C either in medium or in antibody at 1 μg/ml.

when antibody 9-1 was added to a $CD4^+$ MHC class II-restricted, EBV-specific CTL line or to a $CD8^+$ MHC class I-specific CTL line (Table I). Thus antibody 9-1 was only capable of enhancing lysis mediated by $CD16^+$ NK cells and had no effect on lysis mediated by $CD16^-$ CTL.

It was possible that ADCC was responsible for increased lysis in the presence of antibody 9-1. However, neither NALM-1 nor K562 cells have surface CD2 detectable by indirect immunofluorescence and NALM-1 cells do not express Fc receptors whereas K562 cells express FcR_{II} (33, 34). The enhanced lysis induced by antibody 9-1 was directly compared with ADCC by testing the effects of other antibodies known to inhibit the activity of cytolytic cells. When PBL effectors were tested in cytolytic assay against the $CD2^-$ lung carcinoma 2981 cell line, anti-CDw18 antibody 60.3 and anti-CD2 antibodies 9.6 and 35.1 inhibited 9-1 induced lysis but not ADCC, whereas the anti-CD16 Fc receptor antibody FC-2 blocked both activities (Table II). Similar results were obtained by using cultured $CD3^-/CD16^+$ NK cells tested against NALM-1 cells (Table III). Further evidence that antibody 9-1 enhanced killing by directly activating effector cell function, and not by ADCC, was provided by experiments demonstrating that lysis was not increased when target cells were preincubated with antibody 9-1 and washed before the cytotoxic assay (data not shown).

Experiments described above demonstrated that either multivalent cross-linking of CD2 or the formation of complexes between CD2 and CD16 was sufficient to trigger increased $[Ca^{2+}]i$ in NK cells. In cytolytic assays, $F(ab')_2$ of antibody 9-1 did not enhance the lytic activity of either resting or cultured NK cells (Table IV). Simultaneous incubation with $F(ab')_2$ of antibody 9-1 and with a murine

TABLE I

Cytolytic activity of CD3⁺/CD16⁻ cultured alloreactive CTL[a]

CTL Line	Con A	Relevant Target (LCL "DY")		Irrelevant Target (LCL "JH")	
		Medium	Antibody 9-1	Medium	Antibody 9-1
4.47	−	58	47	1	3
	+	47	44	47	45
52	−	57	53	7	5
	+	56	49	19	23

[a] Results represent mean percent specific lysis as measured by ^{51}Cr release assay. Cell line 4.47 is a CD3⁺/CD8⁺/CD16⁻ CTL specific for HLA-B27. Cell line 52 is a CD3/CD4⁺/CD16⁻ HLA-Dw1-restricted CTL specific for an EBV-associated antigen. The EBV-transformed B-lymphoblastoid cell line (LCL) DY but not JH expressed HLA-B27 and HLA-Dw1. The B-lymphoblastoid cell line JH does not express either HLA-B27 or HLA-Dw1. Effector cells were incubated with targets at a 10:1 effector to target ratio for 4 hr at 37°C with antibody (1 μg/ml), Con A (2.5 μg/ml) or medium alone.

TABLE II

Cytolytic activity of peripheral blood lymphocytes: effects of anti-CD16, anti-CDw18, and anti-CD2 monoclonal antibodies on ADCC and lysis induced by antibody 9-1[a]

Antibody	Experiment 1			Experiment 2		
	Medium	Antibody FC-2(CD16)	Antibody 35.1(CD2)	Medium	Antibody 60.3(CDW18)	Antibody 9.6(CD2)
Medium	18	4	18	18	5	13
L6(anti-2981)	47	5	40	33	34	43
9-1(anti-CD2)	87	15	15	78	17	6

[a] Results are expressed as mean percent specific ^{51}Cr release. PBL were assayed for cytotoxicity against 2981 lung carcinoma cells by a 4-hr ^{51}Cr release assay at an effector to target ratio of 100:1. The antibodies were added at a concentration of 10 μg/ml. Antibodies 9.6 and 35.1 do not block binding of antibody 9-1 to effector cells. Antibody L6 used for the ADCC assay binds to target cells but not to human PBL. Antibody 9-1 binds to human PBL but not to the lung carcinoma cell line.

TABLE III

Cytolytic activity of cultured NK cells: effects of anti-CD16, anti-CDw18, and anti-CD2 monoclonal antibodies on lysis induced by antibody 9-1[a]

Stimulus	Medium	Monoclonal Antibody		
		FC-1 (CD16)	60.3 (CDw18)	9.6 (CD2)
Medium	11	9	7	6
Antibody 9-1	34	14	4	4

[a] Results are expressed as mean percent specific ^{51}Cr release. NK cell line EB4.19 (CD3⁻/CD16⁺) was incubated at a 10:1 effector to target ratio with ^{51}Cr-labeled NALM-1 targets. Antibodies were used at a concentration of 1 μg/ml.

TABLE IV

Enhancement of natural killing by antibody 9-1 requires integrity of the Fc domain

Stimulus	Percent Specific ^{51}Cr Release			
	NK cell line[a]	PBL[b]		
		Expt. 1	Expt. 2	
			Medium	Antibody MG21 (IgG3)
Medium	10	3	10	2
Antibody 9-1	35	22	61	65
Antibody 9-1 $F(ab')_2$	4	1	3	7
Antibody 9-1 $F(ab')_2$ + anti-mouse Ig	4	2		

[a] NK cell line EB4.19 (CD3⁻/CD16⁺) was tested against NALM-1 targets in a similar assay in the presence of 9-1 antibody or $F(ab')_2$ at a concentration of 50 μg/ml. A goat anti-mouse Ig antiserum was used at 1/10 dilution.

[b] PBL were assayed for cytotoxicity against ^{51}Cr-labeled 2981 lung carcinoma cells at an effector to target ratio of 10:1 after 4 hr of incubation in the presence of 9-1 antibody or $F(ab')_2$ at a concentration of 10 μg/ml. Antibody MG21 (IgG3) is an isotype control of irrelevant specificity. A rat monoclonal antibody specific for the murine Ig κ-chain was used at 50 μg/ml.

IgG3 immunoglobulin of irrelevant specificity (MG21) did not induce cytolytic activity. Multivalent cross-linking of CD2 with $F(ab')_2$ of antibody 9-1 and a goat anti-mouse Ig antiserum also did not enhance the lytic activity of these cells. These data suggest that an increased $[Ca^{2+}]i$ is not sufficient to trigger the lytic program of NK cells and that interaction with both CD2 and CD16 is required.

DISCUSSION

We have developed a model for probing NK cell activation by using an anti-CD2 monoclonal antibody which cross-links and comodulates CD2 molecules and CD16 Fc receptors. The ability of antibody 9-1 to bind to CD16 may be related in part to its IgG3 isotype. Previous studies using a family of IgG isotype switch variant antibodies with identical avidity for the Thy 1.1 antigen on murine lymphoma SL2 have shown that murine IgG3 antibodies are fivefold more potent than IgG2a and 30-fold more potent than IgG2b in mediating ADCC by human Fc-receptor⁺ NK cells, most likely as a result of a more efficient binding to human CD16 (19). The specific CD2 epitope recognized may represent another possible basis

for the interaction between antibody 9-1 and CD16. Antibodies may bind to this epitope in a way that allows and facilitates an interaction with adjacent CD16 molecules. These two possible explanations for the dual interaction of antibody 9-1 with CD2 and CD16 are not mutually exclusive and both may be important.

Our findings suggest that CD2 is a surface structure capable of transducing signals that increase $[Ca^{2+}]i$ in NK cells. A calcium response was not observed after binding of $F(ab')_2$ of antibody 9-1 but was induced by cross-linking surface-bound 9-1 antibody $F(ab')_2$ or intact 9.6 antibody with a polyclonal anti-murine Ig antiserum. Multivalent cross-linking of CD2 molecules was sufficient to trigger calcium flux, and it appeared that this response does not depend on binding to a particular CD2 epitope. Anti-CD16 antibodies did not induce detectable changes in $[Ca^{2+}]i$ indicating that Fc receptor binding per se does not trigger the calcium response. However, cross-linking surface-bound $F(ab')_2$ of antibody 9-1 with antiglobulin caused a larger increase in $[Ca^{2+}]i$ than cross-linking biotin-conjugated $F(ab')_2$ of antibody 9-1 with avidin, suggesting that bridging and comodulation of CD2 and CD16 may amplify the activation signal. In the case of antibody 9-1, formation of trimolecular complexes between two CD2 molecules and one CD16 molecule appears to mimic the effect of cross-linking CD2 with a murine monoclonal antibody and a polyvalent antiglobulin.

The conditions for inducing an increase in $[Ca^{2+}]i$ in $CD16^-$ CTL and $CD16^+$ NK cells were different. In cultured CTL, a calcium response was observed only when anti-CD2 antibodies 9.6 and 9-1 were used together. Ledbetter et al. (35) have reported that cross-linking of CD2 with antibody 9.6 and a polyclonal goat-anti murine Ig antiserum caused a calcium increase in purified resting T cells. Thus it appears that cross-linking of CD2 molecule represents a critical event required for inducing an increased $[Ca^{2+}]i$ and that this can be accomplished by using pairs of antibodies specific for distinct epitopes (14, 15).

The cascade of events that follow the calcium increase in NK cells has not been elucidated (36). Although it has been reported that a calcium ionophore can induce secretion of lytic granules and release of serine esterase activity by an NK cell line (6), in our hands it failed to enhance cytolytic activity of resting LGL (data not shown). Our experiments indicate that different signals are required for triggering increased $[Ca^{2+}]i$ and for enhancing cytolytic activity. The multivalent cross-linking of CD2 molecules produced by 9-1 $F(ab')_2$ and antiglobulin was capable of inducing increased $[Ca^{2+}]i$ but was not sufficient to enhance cytolytic activity. This indicated that activation of the lytic program by certain CD2 antibodies requires interaction with the CD16 Fc receptor.

Other models of cellular activation through Fc receptors have been described. Cells from the $P388D_1$ macrophage line can lyse chicken erythrocytes in the presence of heteroaggregated antibodies that bridge Fc receptors on effector cells and trinitrophenyl antigen on target cells (37). Heteroaggregates of anti-murine MHC class I and anti-trinitrophenyl antibodies were able to induce conjugate formation but did not facilitate lysis. Taken together, these results suggested a specific role for the macrophage Fc receptor in programming for the lethal hit. Other changes in human NK cells have been described after antibody binding to cell-surface Fc receptors. Human lymphocytes exposed to solid-phase bound immune complexes show flattening and uropod formation (38). Furthermore, Sepharose-bound anti-CD16 antibody has been reported to induce expression of IL-2 receptors and HLA class II molecules on human NK cells (39).

We surmise that Fc receptor triggering in NK cells might induce protein kinase C activity. Nishizuka has proposed that protein kinase C strictly controls calcium homeostasis in various biologic systems (40). We have observed that the kinetics of $[Ca^{2+}]i$ responses triggered by anti-CD2 antibodies are different in CTL and NK cells. In CTL the increase is sustained for at least 15 min, but in NK cells the peak response is followed by a prompt decrease, suggesting the activation of a compensatory mechanism. Support for the possibility that protein kinase C is involved in activation of the lytic program of NK cells has come from a study by Graves et al. who showed that NK cytotoxic factors were released after simultaneous stimulation of PBL with a calcium ionophore and a phorbol ester, whereas either agent alone was ineffective (41). Also, Trinchieri et al. found that preincubation with phorbol esters enhanced spontaneous cytotoxicity of human lymphocytes tested against both NK-sensitive and NK-insensitive targets (42).

Our results and those of other investigators indicate that triggering by anti-CD2 antibodies may be affected by epitope specificity and by the affinity for Fc receptors. It is possible that only certain anti-CD2 antibodies interfere with conjugate formation (22). Binding valency and modulation efficiency may also affect signal transduction, as shown for anti-CD3 antibodies (10). Differences in one or more of these antibody characteristics may explain, for example, why antibodies 9.6 and 35.1 can block enhancement of cytolytic activity by antibody 9-1 and why the 9-1 and 9.6 antibody pair evokes calcium flux in T cells but not the promiscuous cytolytic activity occurring after simultaneous stimulation with the anti-CD2 antibodies $T11_2$ and $T11_3$ (13). On the other hand, we have found that antibodies $T11_2$ and $T11_3$ affect NK cell function with a pattern identical to antibody 9-1 (I. Hellstrom, unpublished observations). Antibodies $T11_2$ (IgG2) and $T11_3$ (IgG3) each evoked a calcium increase in NK cells and induced PBL-mediated lysis of lung carcinoma 2981 target cells that could be blocked by anti-CD16 antibody FC-2 and by anti-CD2 antibody 9.6. These findings suggest that 9-1, $T11_2$, and $T11_3$ antibodies each activate NK cells via a similar mechanism.

It has been proposed that CD2 functions as a surface-adhesion structure involved in cellular interactions (2, 43–45). The natural ligand for CD2 has been shown to be a cell-surface molecule known as LFA-3, a 60- to 70-kDa glycoprotein expressed on a large number of hematopoietic and not hematopoietic cell types (45, 46). It is possible that, upon interaction of CTL and NK effectors with target cells, the natural ligand binds and cross-links CD2 molecules with triggering of a calcium flux in a manner similar to the effect of anti-CD2 antibody binding. In the case of CTL, target cell lysis follows conjugate formation only after recognition of specific antigen by

the T cell receptor or after triggering by anti-CD3 antibodies (1). Our study suggests that target cell lysis by NK cells is triggered by a signal delivered through the Fc receptor. The requirement for two signals would allow activation of the lytic program only when CD16 is triggered by target cell bound IgG and not by soluble immunocomplexes. This model does not exclude the existence of possible NK cell receptors, which might function in parallel with the Fc receptor.

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